Euglena gracilis DNA Dependent RNA Polymerase II: A Zinc Metalloenzyme†

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ABSTRACT: Zinc is essential for cellular proliferation. Zinc deficiency of *Euglena gracilis* results in arrest of cell division and deranges nucleic acid and protein metabolism pointing to a decisive role of zinc in transcription and translation. We have, therefore, investigated the role of zinc in the function of the DNA-dependent RNA polymerases of this organism. Two RNA polymerases from zinc sufficient organisms were purified first by affinity chromatography on a DNA cellulose column and subsequently separated on diethylaminoethyl (DEAE)-Sephadex A-25. The two fractions were characterized as polymerase I and II by their elution pattern from DEAE-Sephadex and sensitivity to α-amanitin. RNA polymerase II has a provisional molecular weight of 700 000 and contains an average of 2.2 g-atoms of zinc per mol of enzyme, but not Mn, Cu, or Fe, as measured by microwave emission spectroscopy. Chelating agents, such as 1,10-phenanthroline, 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, and lomofungin, inhibit activity. In contrast, the nonchelating analogues, 1,7- and 4,7-phenanthroline, do not affect activity. Inhibition by 1,10-phenanthroline is instantaneous and fully reversible by dilution. 1,10-Phenanthroline also inhibits RNA polymerase I, suggesting a role of zinc in its function. The demonstration that RNA polymerase II is a zinc enzyme indicates the involvement of zinc in eukaryotic RNA synthesis and serves as a further basis for the definition of the role of this element in eukaryotic cell growth, division, and differentiation.

Zinc is present in and essential to the growth and proliferation of cells from all phyla (Vallee, 1959, 1976). The unicellular eukaryote, *Euglena gracilis,* has served us to examine both the biological manifestations and biochemical basis of this requirement. The organism can be grown in suspension cultures with excellent yields and is highly responsive to changes in the zinc content of its growth medium (Price and Vallee, 1962). The consequences of zinc deficiency on its growth, morphology, composition, and cell cycle have been reported (Wacker, 1962; Falchuk et al., 1975a,b). Characteristically, the content of DNA, amino acids, polyphosphates, Mn, Mg, Ca, Fe, Cr, and Ni of zinc deficient cells increases, while that of Zn, RNA, and proteins decreases (Wacker, 1962; Falchuk et al., 1975a). Such results provide substantial evidence for the involvement of zinc in biochemical processes essential for both transcription and translation. Moreover, they raise questions regarding the specific processes whose derangements might be responsible for the growth arrest which is the universal result when proliferating cells are deprived of zinc.

In order to define possible roles of zinc in both transcription and translation, to identify the loci of biochemical lesions in zinc deficient *E. gracilis,* and to correlate them with their cellular consequences, we have initiated systematic investigations of the enzymes involved in the polymerization of RNA and DNA. We here report the isolation of the DNA-dependent RNA polymerase II from zinc sufficient *E. gracilis.* The enzyme has been purified to homogeneity and is a zinc metalloenzyme, providing direct evidence for the involvement of zinc in the nucleic acid metabolism of this eukaryote.

Materials and Methods

Euglena gracilis, strain Z, was grown in the dark at 22 °C in media previously described (Price and Vallee, 1962) but in addition, containing 10^{-4} M methionine. When the cultures reached a cell density of approximately 2–3 × 10^6 organisms/ml (the midlog phase of growth), the cells were centrifuged at 150*g* for 5 min. The cell pellets were washed with ice cold buffer in 0.05 M Tris-HCl,[1] pH 8.0, 10^{-3} M 2-mercaptoethanol, and stored at −70 °C. Under these conditions, storage for 3 months did not affect RNA polymerase activity.

Assay for DNA-Dependent RNA Polymerase. Metal-free water was used throughout. Unlabeled nucleotide triphosphates (ATP, CTP, GTP, and UTP) were obtained from Sigma (Sigma Chemical Co., St. Louis, Mo.).

Stock solutions of 2.5 mg/ml of ATP, CTP, and GTP were prepared and stored at −20 °C. [^{3}H]UTP (17 Ci per mM) was obtained from New England Nuclear Corp., Boston, Mass. Calf thymus, *Escherichia coli,* salmon sperm, and *Clostridia perfringens* DNA (Sigma Chemical Co.) were denatured by heating at 100 °C for 15 min in 0.01 M NaCl. A solution of dithiothreitol, 3 mg/ml (Calbiochem., San Diego, Calif.), was prepared fresh daily. Stock solutions of 1 M $MgCl_2$ and 0.1 M $MnCl_2$ were prepared from spectroscopically pure materials (Johnson Matthey Chemicals, Ltd., London).

RNA polymerase activity was measured by the conversion of ^{3}H-labeled nucleotide triphosphate into an acid-insoluble product. The total volume of the standard assay was 0.1 ml. The assay solution contained 10 μmol of Tris-HCl (pH 8.0), 1 μmol of $MgCl_2$, 0.2 μmol of $MnCl_2$, 0.1 μmol of dithiothreitol, 5 × 10^{-3} μmol of each GTP, CTP, and ATP, 5 μCi of [^{3}H]UTP, and 20 μg of heat-denatured, calf-thymus DNA. The assay mixture was incubated for 15 min at 30 °C in an Exatherm P5 Electronic Julabo bath. The enzymatic reaction

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetate; OP, 1,10-phenanthroline.

was terminated by cooling the sample in an ice bath. A 75-μl aliquot from each assay mixture was adsorbed onto a Whatman-1 paper disc, 2.4 cm diameter (Bollum, 1968). Each filter was washed five times with 10 ml of cold 5% Cl_3CCOOH and 1% sodium pyrophosphate, then placed into a 1:1 mixture of ethanol–ether, and allowed to dry under a heat lamp for 10 min. After drying, the filters were counted with an efficiency of 40% using 10 ml of Econofluor (New England Nuclear Corp.) in a LS-335 Beckman scintillation counter. Protein concentration was measured by the method of Lowry. RNA polymerase was stored in 50% glycerol in 2-ml vials at −70 °C in a liquid nitrogen tank (Cryogenics East, Inc.). Under these conditions, activity remained constant for several months. DNA-dependent DNA polymerase activity was determined by measuring the conversion of [^{3}H]TTP into acid-insoluble products as described by Lehman et al. (1958).

DNA-Dependent RNA Polymerase Purification. All preparative work was carried out at 4 °C. A 15-g aliquot of frozen cells was suspended in 65 ml of cold buffer A, 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM mercaptoethanol. For each gram of cells, 1 g of glass beads (Superbright, 3M Corporation, Minn.) was added to aid in disrupting the cells. Before use for cell disruption beads were treated by soaking in 2 N HCl overnight, followed by extensive washing in metal-free water and cooling overnight. Cells were broken manually for 15 min with a mortar and pestle. The mortar and pestle had been cooled overnight at −20 °C. The temperature of the cellular mixture was kept between 0 and −5 °C by frequently immersing the mortar in an acetone–dry ice bath throughout the entire procedure. Ammonium sulfate, final concentration of 0.5 M, was added to the mixture when its consistency was soft and creamy. The resultant viscous solution was homogenized twice for 15 s in a Virtis 45 homogenizer at 40 000 rpm. The homogenizer flask and blades also were precooled at −20 °C and the solution was maintained at or below 0 °C by placing the homogenizer flask in an ice bath during the homogenization. The viscosity of the extract decreased such that the solution could be delivered easily from a Pasteur pipet. The resultant, nonviscous solution was centrifuged for 20 min at 48 000*g* and the supernatant saturated with solid ammonium sulfate up to 50% saturation. The mixture was centrifuged for 10 min at 48 000*g* and the pellet dissolved in 16 ml of buffer B, 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1% Triton X-100, 10% glycerol. The dissolved fraction was loaded onto a Sephadex G-25 column, 6 × 25 cm, and eluted with buffer B. The protein fractions containing RNA polymerase activity, still bound to DNA at this stage of purification, were collected and diluted with buffer B to a conductivity of 2 mmhos. To obtain enzyme free of DNA, the active Sephadex G-25 fractions were pooled and precipitated with 1% of protamine sulfate (Sigma Chemical Co., St. Louis, Mo.) in 0.01 M Tris-HCl (pH 7.4) buffer. Protamine sulfate was added, resulting in a final ratio of 1:24 (v/v). The solution was stirred for 20 min and the resulting pellet was collected by centrifugation of 48 000*g* for 10 min. To remove excess protamine sulfate, the pellet was resuspended and washed in buffer composed of 0.1 M magnesium acetate, 0.05 M Tris-HCl, 0.02 M mercaptoethanol. After 20 min of stirring with magnesium acetate buffer, the precipitate was collected once again by centrifugation at 48 000*g* for 5 min. RNA polymerase was then dissociated from the DNA and solubilized by stirring for 10 min with 10 ml of buffer C, 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 0.1 M ammonium sulfate, 10 mM mercaptoethanol, 20% glycerol, 0.5% Triton X-100. Centrifugation at 48 000*g* for 5 min yielded a supernatant which contained the RNA polymerase. The resulting solution was dialyzed for 4 h in buffer D, 20 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 0.1 M NaCl, 10 mM mercaptoethanol, 10% glycerol, 100 μg/ml bovine albumin, in preparation for DNA cellulose affinity chromatography.

DNA cellulose was prepared according to the method of Alberts et al. (1968). Munktells 410 Cellulose, 3 g (Bio-Rad Laboratories, Richmond, Calif.), was suspended in 9 ml of denatured, calf-thymus DNA in 0.01 M Tris-HCl (pH 7.4) and 0.01 M EDTA. The concentration of DNA was 4 mg/ml. The resultant thick paste was dried overnight at room temperature and the remaining water was removed by lyophilization. About 20 volumes of 0.001 M EDTA, 0.05 M Tris-HCl, pH 7.9, buffer was added to the dry powder and left in the cold room for a day. After three washes to remove free DNA, the DNA cellulose was stored as a frozen suspension in Tris–EDTA buffer containing 0.15 M NaCl. About 50% of the total DNA was bound to the cellulose. A column of 0.5 × 3 cm was packed with the resultant DNA cellulose and equilibrated with 10 ml of buffer D. The flow rate was maintained at 2 ml per h. The enzyme was loaded on the column and washed with 0.1 M NaCl buffer. Fractions of 0.2 ml were collected and the 280-nm absorbance profile of the eluate was obtained on an ISCO Model UA-5 recorder. Employing buffer D all proteins which did not bind to DNA were removed from the column with 0.1 M NaCl and RNA polymerase was eluted with 0.6 M NaCl.

The enzyme obtained from the DNA cellulose column was dialyzed in buffer E, 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 50 mM ammonium chloride, 0.1 mM EDTA, 20% glycerol, 10 mM mercaptoethanol, for 3 h. The enzyme was then centrifuged at 48 000*g* for 5 min and loaded on a 0.5 × 11 cm column of DEAE-Sephadex A-25. The flow rate was maintained at 6 ml/h; the column was washed with 10 ml of equilibration buffer E to collect fractions of 0.2 ml. The enzyme was eluted by step gradients with 0.15 and 0.35 M ammonium chloride in buffer E.

Effect of α-Amanitin on RNA Polymerase Activity. The sensitivity to α-amanitin of activities of the two polymerase fractions obtained on DEAE-Sephadex chromatography was examined at concentrations ranging from 0.01 to 100 μg per ml of α-amanitin. Each assay mixture contained 10 μg of protein from the DEAE-Sephadex fractions. Assays were performed under standard conditions.

Requirements for RNA Synthesis by RNA Polymerase II from E. gracilis. The requirements for the incorporation of nucleotide triphosphates into RNA were investigated, including dependence of RNA polymerase activity on time of incubation, pH, concentration of enzyme, DNA template, nucleotide substrate, manganese, and magnesium.

The effect of DNase and RNase on the product of the reaction was examined as part of the criteria required to establish the enzyme as an RNA polymerase. Following a 30-min incubation period of 40 μg of enzyme, 20 μg of either RNase or DNase (Sigma Chemical Co., St. Louis, Mo.) was added to the incubation mixture, and their effects on [^{3}H]UMP incorporation were assayed 30 min later.

Molecular Weight Determination and Purity of the Enzyme. The homogeneity and molecular weight of the enzyme were determined from four different preparations of *E. gracilis* RNA polymerase (fraction B, DEAE-Sephadex A-25) using 25-μg samples under nondenaturing conditions with gel electrophoresis in 5% acrylamide, and 45 μg in 0.1% sodium dodecyl sulfate gel, respectively (Laemmli, 1970). Standards of reference in gel electrophoresis included 10 μg of highly pu-

TABLE I: Purification of DNA-Dependent RNA Polymerases.[a]

Fraction	Total Protein (mg)	Specific Act. (units[b]/mg)	Total Act. (nmol)	Yield (%)
I. Crude extract	3550	0.5	1775	100
II. Supernatant I	500	3.5	1770	100
III. Ammonium sulfate	346	5.04	1743	100
IV. Sephadex G-25	200	10.0	2000	77
V. Protamine sulfate	18	10.4	187	10.5
VI. DNA cellulose	2.0	94.8	189.6	10.6
VII. DEAE-Sephadex A-25				
A. 0.15 M NH_4Cl	0.3	2.0	0.6	0.3
B. 0.35 M HN_4Cl	1.2	140.0	168	9.4

[a] Fifteen grams of cells used. [b] One unit = incorporation of 1 nmol of [^{3}H]UMP in 15 min at 30 °C.

rified, homogeneous *E. coli* RNA polymerase (Boehringer Mannheim) and 10 μg of albumin. Electrophoreses were carried out for 4 h at 4 °C with a constant current of 4 mA per tube. Subsequently, the gels were removed from each tube and stained with 0.5% Coomassie brilliant blue in 5% methanol and 7% glacial acetic acid to define the subunits of the enzyme.

Densitometric analysis of each of the stained subunits was performed in a Gilford spectrophotometer 240 at 500 nm. The height and density of each band were recorded on a Heathkit chart recorder Model 1R-18 M. The mobilities of the *E. coli* RNA polymerase and albumin bands were plotted as a function of their known molecular weights and the resultant standard curve served to determine the molecular weight of *E. gracilis* RNA polymerase. For each subunit the area under the profile was established also in order to determine the proportional amount of each band in each preparation.

Metal Content. All glassware and pipets utilized in the preparation of the enzyme were soaked in a 50:50 mixture of nitric and sulfuric acids and washed extensively with metal-free distilled water in order to avoid external metal contamination.

The 2n, Mn, Cu, and Fe content of the purified enzyme (RNA polymerase II) was determined by microwave excitation emission spectroscopy (Kawaguchi and Auld, 1975), a method capable of measuring metal concentration quantitatively in the picogram range. Hence, the metal content of microgram quantities of protein can be determined as has been described (Kawaguchi and Vallee, 1975).

Aliquots of 100 to 450 μg of purified DEAE-Sephadex A-25 enzyme (RNA polymerase II) in 0.2 ml of buffer were dialyzed against 200 ml of metal-free 0.05 M Tris, pH 8.0 at 4 °C, with five changes of dialysis buffer. Enzyme activity was assayed before and after dialysis. Following dialysis, the enzyme was diluted 1:1 with 10 mM KCl. The addition of 4 to 10 mM KCl to the samples enhances the intensity of the metal spectra and eliminates or suppresses interference (Kawaguchi and Vallee, 1975; Kawaguchi and Auld, 1975). Five -microliter aliquots of these enzyme solutions, containing from 0.5 to 3 μg of protein, were used for metal analysis. Four different enzyme preparations were analyzed in triplicate.

Inhibition by 1,10-Phenanthroline. The chelating agent 1,10-phenanthroline (OP) has proven exceptionally suitable to study the inhibition of zinc metalloenzymes (Vallee and Wacker, 1970). To determine its effect on *E. gracilis* RNA polymerase II, the effect of OP concentration and time of preincubation on enzyme activity and the reversibility of inhibition were all studied. A stock solution of OP, 10^{-2} M, pH 7.5, was diluted variously to range from 10^{-2} to 10^{-7} M. The concentrations of template, nucleotide, and other components were standard in all assays. Throughout Mg^{2+} was the only activating cation. In one set of experiments, 10 μg of enzyme was added directly to an assay mixture containing different concentrations of the chelating agent. In other experiments, enzyme was preincubated with OP from 0 to 60 min and aliquots were pipetted into the assay mixture containing concentrations of inhibitor sufficient to maintain that of the preincubation mixture. To ascertain possible reversibility of the OP inhibition, aliquots of enzyme preincubated with OP for 0, 15, 30, and 60 min also were diluted up to tenfold with buffer prior to assay. The effects of the nonchelating isomers, 1,7-, or 4,7-phenanthroline, in the concentration range from 1 to 3×10^{-4} M were determined also.

The effect of OP on RNA polymerase activity of the active protein fraction eluted with 0.15 M NH_4Cl from the DEAE-Sephadex A-25 also was investigated. In these experiments 10 μg of enzyme was incubated with 10^{-3} M OP followed by assay of its activity.

Inhibition Studies with Other Chelating Compounds. The effect of other chelating agents on activity was studied as a function of their concentrations. Stock solutions of 8-hydroxyquinoline-5-sulfonic acid, EDTA, α,α′-bipyridyl, or 8-hydroxyquinoline, all 5×10^{-2} M, were diluted with metal-free water to prepare dilutions ranging from 10^{-6} to 10^{-2} M, adjusted to pH 8. Assays were performed with 10 μg of enzyme and under standard conditions. Magnesium was the activating metal in all cases.

Inhibition of RNA Polymerase by Lomofungin. The antibiotic lomofungin[2] from *Streptomyces lomodensis* inhibits RNA synthesis in yeast. Like 8-hydroxyquinoline, this compound chelates divalent cations like zinc, magnesium, and manganese (Pavletich et al., 1974). Consequently, we have investigated its effect on the activity of RNA polymerase II from *E. gracilis*. Sodium hydroxide (0.025 M) was added dropwise to 2 mg of lomofungin until the material dissolved. The volume was adjusted to 2 ml and aliquots containing 5 to 100 μg per ml of the inhibitor were incubated with enzyme.

Results

The isolation scheme for RNA polymerases from *E. gracilis* is shown in Table I. The solubilized protein (fraction II) is concentrated with ammonium sulfate and, compared with the crude extract, the specific activity increases tenfold. Gel filtration through Sephadex G-25 gel further increases specific activity and is obligatory for optimal precipitation of the enzyme with protamine sulfate. Protamine sulfate does not alter the specific activity, though nearly 90% of the total activity is lost. However, the enzyme is no longer bound to DNA, as ev-

[2] Gift of Dr. G. Whitfield, Upjohn Company, Kalamazoo, Michigan.

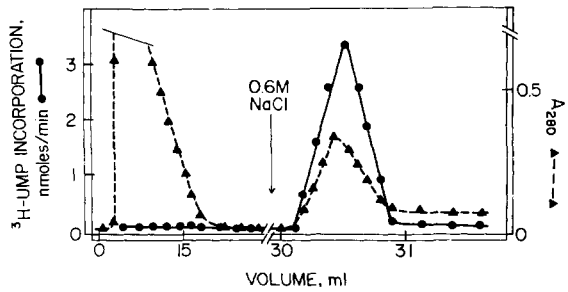

FIGURE 1: Chromatography of *E. gracilis* RNA polymerases on DNA cellulose. Three milliliters of enzyme was solubilized from the protamine sulfate precipitate (fraction V), dialyzed against 500 ml of buffer D, and loaded onto a 0.5 × 3 cm DNA cellulose column. Proteins which do not bind to DNA were removed with 0.1 M NaCl while the RNA polymerase was eluted with 0.6 M NaCl all at a flow rate of 2 ml/h. Two-microliter fractions were assayed for polymerase activity (●) and A_{280} (▲).

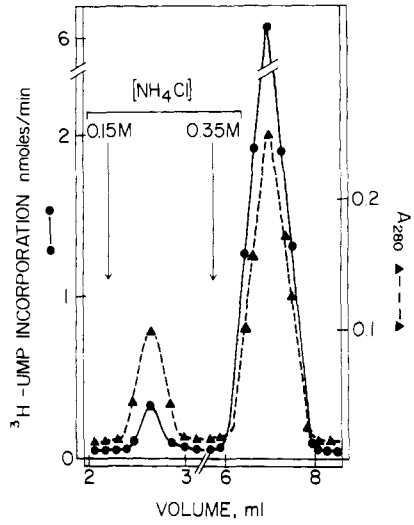

FIGURE 2: Chromatography of *E. gracilis* RNA polymerases on DEAE-Sephadex A-25. DNA cellulose fractions containing RNA polymerase activity were pooled and dialyzed in buffer E. One milliliter was loaded onto a 0.5 × 11 cm column of DEAE-Sephadex A-25 previously equilibrated with this buffer as described in the text, and 0.2-ml fractions were collected. The enzymes were eluted by step gradients with 0.15 and 0.35 M ammonium chloride. Aliquots were assayed for polymerase activity (●) and A_{280} (▲).

idenced by an absolute exogenous DNA requirement for activity, a critical feature which allows the DNA-free enzyme to be purified further by DNA cellulose affinity chromatography. Figure 1 illustrates a typical chromatogram on DNA cellulose. In buffer D most of the protein applied to the column does not bind and appears either in the initial eluate or during washing of the column with 0.1 M NaCl while RNA polymerase activity elutes as a single fraction with 0.6 M NaCl with a specific activity now 190-fold that of the crude extract.

The DNA cellulose fraction containing polymerase activity is resolved further on DEAE-Sephadex A-25. A small active protein fraction (A) elutes with 0.15 M NH_4Cl, while a second major one (B) elutes with 0.35 M NH_4Cl (Figure 2). Neither protein nor activity is detected in the initial or in a 0.6 M NH_4Cl eluate. This procedure increases specific activity of 0.35 M NH_4Cl fraction 280-fold compared with the crude extract. The yield from a typical preparation, using 15 g of cells in 9–10%, with 0.3 and 1.2 mg of enzyme eluted from DEAE-Sephadex A-25 with 0.15 and 0.35 M NH_4Cl, respectively.

The two DEAE-Sephadex A-25 fractions differ in regard

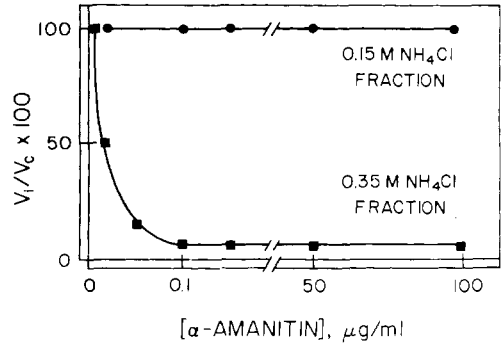

FIGURE 3: The effect of α-amanitin on the activities of RNA polymerases I and II. Ten-microgram aliquots of enzymes eluted from the DEAE-Sephadex with 0.15 and 0.35 M NH_4Cl were incubated for 15 min at 30 °C with concentrations of α-amanitin ranging from 0.01 to 100 μg/ml. V_i is the velocity in the presence of α-amanitin and V_c is the velocity in its absence.

to their sensitivity to α-amanitin (Figure 3). The activity of the first DEAE-Sephadex fraction is not inhibited by α-amanitin at concentrations up to 100 μg per ml. This indicates that this enzyme is an RNA polymerase I. In contrast, increasing concentrations of α-amanitin progressively decrease and at 0.1 μg/ml nearly abolish activity of the second DEAE-Sephadex fraction, typical of an RNA polymerase II (Chambon, 1974).

Characterization and Properties of the DNA-Dependent RNA Polymerase II of E. gracilis. *E. gracilis* DNA dependent RNA polymerase requires DNA, four ribonucleotide triphosphates, and metal ions (Mg^{2+} or Mn^{2+}) for activity. The dependence on exogenous template becomes apparent only after the protamine sulfate precipitation step (fraction V).

The product of the complete enzymatic reaction is sensitive to RNase but not to DNase digestion. Enzyme activity varies according to the source and physical state of the template. Activity is two to three times greater with denatured than with native templates from calf thymus, salmon sperm, and *Clostridia perfringens.* In contrast, both native and denatured DNA from *E. coli* serve equally well. Four nucleotide triphosphates are an essential requirement for activity. Omitting any one of these or substituting deoxyribonucleotides curtails enzymatic activity. Under the present assay conditions, RNA polymerase II, like analogous enzymes, is inactive in the absence of either manganese or magnesium. Manganese activates the RNA polymerase over a narrow concentration range and is maximal at 2 mM. At higher manganese concentrations, enzyme activity decreases. Activity with magnesium is maximal at 10 mM, but (in contrast to manganese) increases even up to 100 mM do not inhibit enzyme activity.

The incorporation of UMP depends linearly on the time of incubation up to 30 min, indicating that the substrate and template concentrations are not limiting in assays performed over this concentration range. Activity is linearly proportional to enzyme concentration over the range from 10 to 25 μg. Enzyme activity increases as the substrate concentration is raised from 1 to 10 μM but does not change further up to 50 μM. No substrate inhibition is noted. The K_m for UMP incorporation is 5 μM as calculated from Lineweaver–Burk plots. Enzymatic activity also increases as the DNA concentration is raised from 2 to 20 μg/0.1 ml assay, with a plateau reached at 20 μg/0.1 ml. Hence, assays were performed with 50 μM and 20 μg/0.1 ml nucleotide triphosphate and template concentration, respectively. Polymerase II activity is optimal at pH 7.9 and falls off drastically at lower and higher pH's.

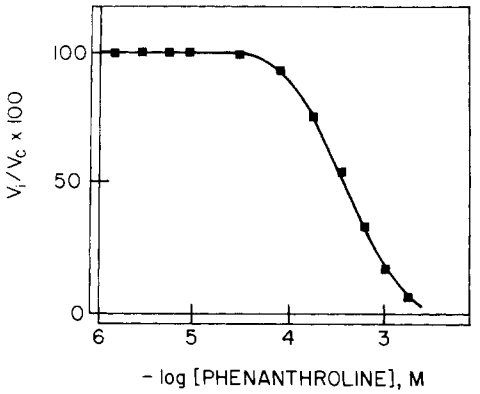

FIGURE 4: The effect on enzyme activity of incubating RNA polymerase II with 1,10-phenanthroline (OP), at concentrations between 10^{-6} and 5×10^{-3} M. V_i is the velocity in the presence of inhibitor and V_c is the velocity in its absence. Ten micrograms of enzyme was preincubated with OP and assays were performed at 30 °C for 20 min. Mg^{2+} (10 mM) served as the activating metal.

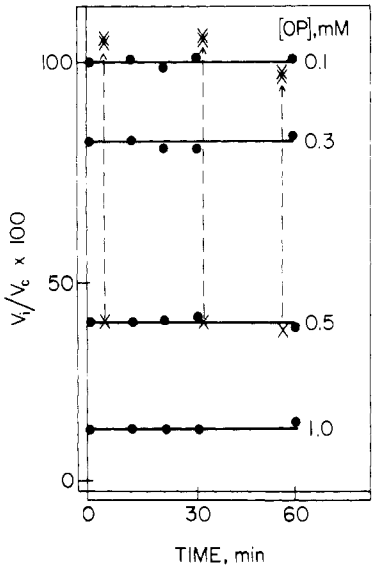

FIGURE 5: The effect on the activity of RNA polymerase II of varying the time of preincubation with 1,10-phenanthroline (OP). Ten micrograms of enzyme was preincubated with OP at concentrations ranging from 0.1 to 1.0 mM. The activities of aliquots of enzyme preincubated with OP were assayed at 30 °C for 15 min at various time intervals (●). The OP concentration in each assay was identical with that in the preincubation mixture. At 5, 30, and 60 min (X), aliquots of enzyme were diluted with 0.1 M Tris (pH 8.0)–0.1 mM dithiothreitol buffer and the activity was assayed. The resultant activity (XX) was corrected for dilution. In all cases, Mg^{2+} (10 mM) was the only activating metal present.

Homogeneity and Molecular Weight Determination. Neither of the proteins eluted from DEAE-Sephadex A-25 contains DNA dependent DNA polymerase, as evidenced by lack of incorporation of deoxyribonucleotide triphosphates into acid-insoluble DNA. On polyacrylamide gel at pH 8.3, RNA polymerase II moves as a single cationic band, similar to *E. coli* RNA polymerase analyzed under the same conditions. It separates into five bands in 0.1% sodium dodecyl sulfate gels. Densitometry of these bands in stained gels indicates a ratio of 1:1:1:1:(1–2) with calculated molecular weights of 208 000, 160 000, 133 000, 83 000, and 64 000. The combined weights suggest an approximate molecular weight of 700 000 daltons for the holoenzyme.

Effect of Metal Chelating Agents on Enzyme Activity. The functional role of metals, other than Mg or Mn, in the activity of RNA polymerase II was ascertained with various chelating agents. Enzyme activity is inhibited by OP, 8-hydroxyquinoline-5-sulfonic acid, 8-hydroxyquinoline, EDTA, and α,α'-bipyridyl in concentrations ranging from 10^{-4} to 10^{-2} M (Table II). OP (5×10^{-3} M) also inhibits polymerase I.

TABLE II: Effect of Metal Chelating and Nonchelating Agents on DNA-Dependent RNA Polymerase II Activity.

Agent	Concn ($M \times 10^3$)	Inhibition (%)
1,10-Phenanthroline	0.1	4
	0.3	42
	5.0	100
8-Hydroxyquinoline	0.1	2
	3.0	40
8-Hydroxyquinoline-5-sulfonate	0.2	15
	0.5	22
	5.0	40
α,α'-Bipyridyl	4.0	43
EDTA	0.1	7
	1.0	75
	10.0	100
Lomofungin	*a*	11
	b	93
1,7-Phenanthroline	0.1	0
	0.3	0
4,7-Phenanthroline	0.1	0
	0.3	2

[a] Concentration, 20 μg/ml. [b] Concentration, 100 μg/ml.

Inhibition of polymerase II by OP was studied in detail. Addition of 10^{-4} to 5×10^{-3} M OP to the assay mixture progressively inhibits enzyme activity, the latter concentration completely inactivating the enzyme (Figure 4). OP inhibition is instantaneous and completely reversible on dilution of the chelating agent with buffer (Figure 5). In the presence of 10 mM Mg^{2+}, the pK_I for OP inhibition is 3.4. No inhibition is observed at concentrations of OP below 10^{-4} M.

The nonchelating isomers, 1,7- or 4,7-phenanthroline, 3×10^{-4} M, do not inhibit enzyme activity. Under the same conditions, the 1,10 isomer causes 42% inhibition of the enzyme. EDTA, 8-hydroxyquinoline, and 8-hydroxyquinoline-5-sulfonic acid also inhibit polymerase II (Table II). EDTA, below 10^{-4} M, does not inhibit polymerase II activity but progressively inactivates above this concentration and is complete at 5×10^{-3} M. The enzyme is inhibited by 8-hydroxyquinoline-5-sulfonic acid from 10^{-4} to 10^{-2} M and by 8-hydroxyquinoline from 10^{-3} to 10^{-2} M. The low solubility of these agents precluded complete inhibition. Lomofungin (100 μg/ml) and α,α'-bipyridyl (5×10^{-3} M) inhibit the enzyme by 93 and 50%, respectively.

Metal Content. The activity of the enzyme samples used for metal analysis remained constant during the repeated dialyses required to remove metal contaminants. The metal content of RNA polymerase II as determined by microwave emission is shown in Table III.

The zinc content in the 5-μl samples analyzed ranged from 0.185 to 0.251 μg of zinc/mg of protein, while copper and iron are absent and manganese is present in only trace amounts. Therefore, based on a provisional molecular weight of 700 000, RNA polymerase II contains 2.2 g-atoms of zinc per mole of enzyme (Table III).

Discussion

In *E. gracilis,* zinc deficiency increases the content of DNA and amino acids while decreasing that of RNA and protein

TABLE III: *Euglena gracilis* RNA Polymerase II Metal Content.

Metal	Protein (mg/ml)	Metal Content (μg/mg of Protein)	Metal Content (g-atoms)[a]
Zn	0.10	0.204	2.2
	0.10	0.185	2.0
	0.18	0.203	2.2
	0.45	0.251	2.6
Mn	0.10	0.015	0.19
Cu	0.10	0.0	0.0
Fe	0.10	0.0	0.0

[a] Per molecular weight 700 000.

(Wacker, 1962; Wacker et al., 1965; Prask and Plocke, 1971). These observations provided the first evidence that zinc is essential to molecular processes underlying either transcription, translation, or both. We have continued to study *E. gracilis* to investigate this biochemical role of zinc systematically and to determine the metabolic lesions which result when this element becomes limiting to the processes critical to cell growth and division. Morphologic studies have been performed to identify possible effects of zinc deficiency on the structure of the resultant cells and of the organelles involved in the metabolism of nucleic acids and proteins. Zinc deprivation does not alter the ultrastructure of the nucleus, ribosomes, Golgi apparatus, or mitochondria (Falchuk et al., 1975a). The doubling of the DNA content of zinc deficient cells remains one of the striking demonstrable abnormalities and suggests derangements in the biochemical processes associated with its metabolism. Flow microfluorometry has been utilized to investigate the DNA metabolism of both zinc sufficient and deficient *E. gracilis* cells. Under conditions of random growth, the proliferative arrest of zinc deficient cells manifests a premitotic block localized to S/G2. Synchronized cells can be blocked at G1 as well (Falchuk et al., 1975b). Though zinc is essential for the function of DNA polymerase of *E. coli* (Slater et al., 1971) and indirect evidence suggests this to be true for the enzyme from *E. gracilis* as well (McLennan and Keir, 1975), the results of our studies of DNA metabolism led to the conclusion that the limiting steps leading to the abnormalities of the cell cycle and the consequent proliferative arrest cannot be the consequence solely of impaired DNA synthesis.

The diminution of the RNA content generates obvious questions regarding derangements in its synthesis or its subsequent catabolism and the accumulation of peptides and amino acids followed by decreases in protein content further focuses on functional consequences at the translational level. Such derangements could potentially be responsible both for the observed blocks of the cell cycle and the proliferative arrest since on-going RNA and protein synthesis are required for G1, S, and G2. Alterations in their synthesis could then block the cell cycle at each of these stages (Gelfant, 1966; Avanzi et al., 1969; Epifanova and Terskikhi, 1969; van't Hof, 1974). Accordingly, we are inspecting the role of zinc in *E. gracilis* DNA dependent RNA polymerases in detail as one possible basis for the observed chemical lesions since these enzymes play a central role in both transcription and translation.

Detailed studies of the catalytic role of zinc in *E. gracilis* RNA polymerases require the preparation of highly purified, physicochemically homogeneous enzyme in quantities sufficient to establish its metal content, a problem that had proved difficult for other eukaryotic polymerases (Cold Spring Harbor Symposium, 1970). Assisted in some measure by recent methodological advances in their isolation (Cold Spring Harbor Symposium, 1970), we have succeeded in purifying RNA polymerase II of *E. gracilis* to homogeneity (Table I, Figures 1 and 2). The enzyme meets all criteria for the definition of DNA dependent RNA polymerases: the activity is not manifest in the absence of exogenous DNA or of four nucleotide triphosphates, and the product of the reaction is digested by RNase but not by DNase. The activity requirements of the enzyme resemble those for other eukaryotic RNA polymerases II, including its complete inhibition by low doses of α-amanitin (Lindell et al., 1970; Chambon, 1974), an optimal pH near 7.9, and activation by Mn^{2+} or Mg^{2+}. The degree of activation with Mn^{2+} exceeds that with Mg^{2+}, as has been reported for the polymerases from maize seedlings, coconut endosperm, yeast, and fungi (Chambon, 1974). The K_m for UMP, 0.005 mM for the *E. gracilis* enzyme, compares with 0.02 mM for the A_1 and B calf thymus enzymes (Chambon, 1974). The molecular weight of *E. gracilis* RNA polymerase II is similar to that of other eukaryotes, typically between 500 000 and 700 000 (Biswas et al., 1975). This analogy extends to the five bands observed on polyacrylamide gel electrophoresis, though both fewer and larger numbers of subunits have been reported for RNA polymerases from other species (Chambon, 1974; Biswas et al., 1975). The bands on polyacrylamide gel are thought to reflect six subunits, two of which have identical molecular weights.

Purified RNA polymerase II is inhibited by OP, analogous to other DNA and RNA polymerases from viruses, *E. coli, E. gracilis,* sea urchin, liver, and yeast (Slater et al., 1971; Scrutton et al., 1971; Springgate et al., 1973; Coleman, 1974; Auld et al., 1974a–c, 1975, 1976; McLennan and Keir, 1975), suggesting that in all these instances zinc is critical to the function of these enzymes.

However, proof for this inference could not be obtained until quantitative metal analyses became possible. Such analyses posed major problems since only microgram quantities of enzyme were available for study. However, we have recently designed methods capable of quantitatively determining 10^{-14} g of zinc and other metals in microgram quantities of enzyme to solve similar problems in studies of the RNA-dependent DNA polymerases from oncogenic type C viruses. The details and their applications to biologic systems have been presented (Kawaguchi and Vallee, 1975; Kawaguchi and Auld, 1975; Auld et al., 1975).

We have now successfully utilized microwave excitation emission spectroscopy to demonstrate that RNA polymerase II is a zinc enzyme. Analysis of its zinc content was performed on 0.5- to 3-μg samples of enzyme containing from 1×10^{-10} to 5×10^{-10} g of zinc and no other transition metals (Table III). On the basis of these measurements, the *E. gracilis* RNA polymerase II contains 2.2 g-atoms of zinc per provisional molecular weight of 700 000 (Table III).

General considerations regarding the variables involved in determining the absolute metal content of alkaline phosphatases were discussed recently (Bosron et al., 1975) and are pertinent to the present case. In particular, the zinc content of the RNA polymerase II has been expressed in units of g-atoms per mol of enzyme; hence it is dependent on the accuracy of the molecular weight, which was calculated from the subunit stoichiometry of the enzyme. Possible ambiguities in the assessment of the exact subunit stoichiometry of polyacrylamide gel electrophoresis could alter the present molecular weight of enzyme and, hence, the metal stoichiometry. The precise molecular weight of the enzyme, and, as a consequence, the numbers of g-atoms of zinc present must remain provisional

until the relevant data are obtained by, e.g., ultracentrifugation.

The inhibition studies with chelating agents, particularly OP (Figures 4 and 5), provide insight into the possible functional role of zinc in this enzyme. The inhibition of *E. gracilis* RNA polymerase II with OP is instantaneous; it does not require preincubation for any period of time. Preincubation alters neither the degree nor the rate of inhibition (Figure 5). Further, on dilution of the reaction mixture, the inhibition is fully reversible. Activity returns to that expected for the concentration of OP subsequent to dilution. Such data are characteristic for the formation of a mixed complex between a metalloenzyme and OP. In contrast the removal of the metal generally manifests as a time-dependent process (Vallee and Wacker, 1970). Instantaneous time-independent OP inhibition has previously been observed only for horse and human liver alcohol dehydrogenase (Vallee and Hoch, 1957; Sytkowski and Vallee, 1976).

In addition to Zn, the activities of RNA polymerases require Mg^{2+} (or Mn^{2+}). Thus, at least two metal species are required for function. Even though zinc is an intrinsic component, in the absence of either Mn^{2+} or Mg^{2+} the enzyme is essentially inactive. Though the specific role of metals may differ in the various enzymes, a number of enzymes have similar dual requirements for Zn on the one hand and Mg and Mn on the other, e.g., *E. coli* DNA and RNA polymerases, alkaline phosphatases, and leucine aminopeptidase (Lehman et al., 1958; Chamberlain and Berg, 1962; Plocke et al., 1962; Simpson et al., 1968; Himmelhoch, 1969; Slater et al., 1971; Scrutton et al., 1971; Carpenter and Vahl, 1973; Anderson et al., 1975; Bosron et al., 1975). In zinc alkaline phosphatase, magnesium regulates the mode of binding of zinc and enhances the enzyme activity (Anderson et al., 1975). The functions of magnesium, manganese, and zinc in nucleic acid polymerases have not been detailed in these terms, though roles in substrate, template, or initiator binding have been variously suggested (Englund et al., 1969; Ishihama and Hurwitz, 1969; Chang and Bollum, 1970; Scrutton et al., 1971; Slater et al., 1972).

OP also completely inhibits *E. gracilis* RNA polymerase I, suggesting a role of zinc in its function, consistent with that of this metal in yeast RNA polymerase I (Auld et al., 1976). Its metal composition is being determined.

The demonstration that *E. gracilis* RNA polymerase II is a zinc metalloenzyme confirms the essentiality of this element for RNA synthesis in this organism, and the fact that OP also inhibits RNA polymerase I reinforces this conclusion. This participation of zinc in RNA synthesis, in conjunction with its involvement in other aspects of both RNA (Wacker et al., 1965; Prask and Plocke, 1971) and DNA metabolism (McLennan and Keir, 1975), represents critical steps in deciphering the series of events which may jointly account for its requirement in the growth in *E. gracilis* and other organisms. While attempts to specify a particular enzymatic (or other) step in zinc deficiency which might limit growth would be conjectural at present, a number of alternatives suggest themselves.

Lack of zinc could preclude synthesis of any one or all of these zinc enzymes or result in inactive apoenzyme(s). Further, the possibility must be considered that functional metalloenzymes containing different metals might be generated which could exhibit altered values of K_{cat} or K_m (either for substrate or template). In this regard, the accumulation of Fe, Cr, and Ni which occur in response to zinc deficiency of *E. gracilis* (Wacker, 1962; Falchuk et al., 1975a) could reflect a compensatory mechanism, conceivably designed to overcome a metal-dependent metabolic block. The simultaneous accumulation of Mg and Mn could similarly serve to regulate such metal-substituted RNA polymerases. Certainly the requirements for both Mg and Mn of the corresponding native enzymes are variable though they are all inhibited by high concentrations of Mn (Roeder and Rutter, 1969). Whatever the specific mechanism, the zinc deficient state might alter the relative proportions of existent polymerase activities or induce new variants. A number of observations would tend to support such conjectures. Thus, in the present studies with zinc sufficient *E. gracilis* we have obtained evidence for the existence of RNA polymerases I and II (Figures 2 and 3). Results of on-going studies with zinc deficient *E. gracilis*, on the other hand, already demonstrate the existence of an RNA polymerase species which differs from those of zinc sufficient cells (unpublished observations). Thus, regulatory mechanisms which serve to synthesize or preferentially activate different classes of RNA polymerases appear to be called into play in zinc deficiency. Similar control processes exist during normal growth where in sea urchin (Nemer and Infante, 1965; Emerson and Humphreys, 1970; Roeder and Rutter, 1970), liver (Blatti et al., 1970), *Helianthus tuberosus* (Fraser, 1975), and amoeba (Yagura et al., 1976) the activities of RNA polymerase I and II vary as a function of the stage of development. As a consequence, the classes of RNA synthesized are altered. Such preferential activation or synthesis of the various RNA polymerase species in zinc deficient cells also might result in derangements in RNA metabolism. Further, Pogo et al. (1967) have shown in experiments with *E. coli* RNA polymerase that the relative preponderance of Mg and Mn can modify the nucleotide sequences of the RNA which is the product of the polymerization reaction, a course of events which could obtain during zinc deficiency. One, a combination or all of these processes could ultimately result in the synthesis of altered or abnormal proteins, owing to the RNA species generated and the critical nature to protein synthesis of the RNA nucleotide sequences for, e.g., the binding of messenger RNA and of aminoacyl-tRNA to ribosomes (Ochoa and Mazunda, 1974; Lucas-Lenard and Beres, 1974; Tate and Caskey, 1974). The observations that zinc deficient *E. gracilis* synthesize peptides and/or proteins whose amino acid composition is unusual (Wacker et al., 1965) provides evidence that there could be defects in protein synthesis of zinc deficient *E. gracilis* either during initiation, elongation, or termination. The observation that elongation factor I from rat liver contains functional zinc atoms (Kotsipoulos and Mohr, 1975) is consistent with and could bear importantly on these considerations, though at present it is not possible to discriminate between such alternatives. Clearly, critical experiments to delineate between them are both indicated and feasible.

The present data provide evidence for a role of zinc in transcription and translation of *E. gracilis* underlying the essentiality of zinc in cell division. The demonstration that eukaryotic RNA polymerases I (Auld et al., 1976) and now II are zinc enzymes support the thesis that the element is essential for DNA and RNA metabolism in all phyla (Slater et al., 1971; Scrutton et al., 1971; Springgate et al., 1973; Coleman, 1974; Auld et al., 1974a–c, 1975). The data further suggest that extension of our studies on zinc deficient *E. gracilis* may assist in generalizing the emerging biochemical role of zinc in growth, proliferation, and differentiation.

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